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Isozyme Function of *n*-Alkane-inducible Cytochromes P450 in *Candida maltosa* Revealed by Sequential Gene Disruption*

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An *n*-alkane-assimilating yeast *Candida maltosa* contains multiple *n*-alkane-inducible forms of cytochromes P450 (P450alk), which can be assumed to catalyze terminal hydroxylation of *n*-alkanes in the assimilation pathway. Eight structurally related P450alk genes have been identified. In the present study, the function of four major isoforms of P450alk (encoded by *ALK1*, *ALK2*, *ALK3*, and *ALK5* genes) was investigated by sequential gene disruption. Auxotrophic markers used for the selection of disrupted strains were regenerated repeatedly through either mitotic recombination between heterozygous alleles of the diploid genome or directed deletion of the marker gene, to allow sequential gene disruptions within a single strain. The strain depleted of all four isoforms could not utilize *n*-alkanes for growth, providing direct evidence that P450alk is essential for *n*-alkane assimilation. Growth properties of a series of intermediate disrupted strains, plasmid-based complementation, and enzyme assays after heterologous expression of single isoforms revealed (i) that each of the four individual isoforms is alone sufficient to allow growth on long chain *n*-alkane; (ii) that the *ALK1*-encoding isoform is the most versatile and efficient P450alk form, considering both its enzymatic activity and its ability to confer growth on *n*-alkanes of different chain length; and (iii) that the *ALK5*-encoding isoform exhibits a rather narrow substrate specificity and thus cannot support the utilization of short chain *n*-alkanes.

Cytochromes P450 are heme-containing monooxygenases that are distributed widely among living organisms (1). Higher eukaryotes generally contain multiple forms of cytochrome P450 catalyzing diverse oxidative reactions in the metabolism of a large number of endogenous and xenobiotic compounds. Depending on the induction level and substrate specificity of individual cytochromes P450, cellular metabolic processes are often effected by a specific ensemble of isoforms, making it difficult to distinguish their biological function.

In microorganisms, such a cytochrome P450 multiplicity only rarely can be found. One of the microorganisms harboring a cytochrome P450 multigene family is an *n*-alkane-assimilating yeast *Candida maltosa* (2–6). Resembling the situation in *Candida tropicalis* (7), this yeast species contains *n*-alkane-inducible forms of P450 (P450alk), and eight structurally related P450alk genes belonging to the *CYP52* family were identified. Coupled with NADPH-cytochrome P450 reductase (8), they are assumed to catalyze the terminal hydroxylation of *n*-alkanes, which represents the first and rate-limiting step in the *n*-alkane assimilation pathway. The functions of the *C. maltosa* cytochromes P450 were first apparent from *in vitro* reconstitution of *n*-alkane hydroxylase and NADPH-cytochrome P450 reductase purified from *n*-alkane-grown *C. maltosa* cells (9) and could be confirmed later by *in vivo* CO inhibition studies (10). In addition to these investigations, recent studies revealed the enzymatic characters of some P450alk forms by means of heterologous expression in *Saccharomyces cerevisiae* (11–13). However, investigations about the cellular significance of individual P450alk isoforms for the *n*-alkane assimilation pathway are still fragmental. In particular, it remains to be clarified whether indeed such a P450alk multiplicity is required for the *n*-alkane assimilation pathway of *C. maltosa* and to what extent each of the gene products of eight P450alk genes contributes to the respective *n*-alkane-assimilating phenotype.

Recent development of the genetic engineering systems including host-vector systems as well as the gene disruption method, which enabled us to disrupt stepwise both alleles of a certain gene in the diploid genome by using two selectable markers, have greatly facilitated molecular biological analyses of *C. maltosa* (14–19). As an initial step to address the cellular function of individual isoforms of P450alk, we previously disrupted the first isolated P450alk gene and found that the disrupted stain retained the ability to utilize *n*-alkanes as a sole carbon and energy source because of the *n*-alkane hydroxylation activity of the other P450alk forms. In the present paper we describe the sequential disruption of multiple P450alk genes and show the effect of these disruptions on the *n*-alkane-assimilating property of *C. maltosa*. The results in this paper provide evidence that the difference in substrate specificity among multiple isoforms of P450alk *in vivo* can be detected clearly by using the gene disruption technique even in a non-conventional yeast.

EXPERIMENTAL PROCEDURES

Strains and Media—The *C. maltosa* strains used in this study are listed in Table I. Media for *C. maltosa* were YPD (1% yeast extract, 2% Bacto-pepton, and 2% glucose) and YNB (0.67% Yeast Nitrogen Base (Difco) without amino acids and either 2% glucose or 1% *n*-alkanes), which was supplied with appropriate nutrients. 5-Fluoro-orotic acid

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TABLE I
Candida maltosa strains used in this study

Strain	Parent	Genotype	Relevant phenotype
CHA1[a]	IAM12247	*his5, ade1*	His^-, Ade^-, Ura^+
DA1–44[a]	CHA1	*his5, ade1, alk1::HIS5/alk1::ADE1*	His^+, Ade^+, Ura^+
CHAU1[a]	CHA1	*his5, ade1, ura3*	His^-, Ade^-, Ura^-
DA23–8	CHAU1	*his5, ade1, ura3, alk2-alk3::URA3/ALK2-ALK3*	His^-, Ade^-, Ura^+
DA23–81	DA23–8	*his5, ade1, ura3, alk2-alk3::URA3/alk2-alk3::ADE1*	His^-, Ade^+, Ura^+
DA23–816	DA23–81	*his5, ade1, ura3, alk2-alk3::URA3/alk2-alk3::Δade1*	His^-, Ade^-, Ura^+
DA23–8164	DA23–816	*his5, ade1, ura3, alk2-alk3::Δade1/alk2-alk3::Δade1*	His^-, Ade^-, Ura^-
DA5–6	CHAU1	*his5, ade1, ura3, alk5::URA3/ALK5*	His^-, Ade^-, Ura^+
DA5–61	DA5–6	*his5, ade1, ura3, alk5::URA3/alk5::ADE1*	His^-, Ade^-, Ura^+
DA5–611	DA5–61	*his5, ade1, ura3, alk5::ADE1/alk5::ADE1*	His^-, Ade^+, Ura^-
DA15–2	DA5–611	*his5, ade1, ura3, alk5::ADE1/alk5::ADE1 alk1::URA3/ALK1*	His^-, Ade^+, Ura^+
DA15–23	DA5–2	*his5, ade1, ura3, alk5::ADE1/alk5::ADE1 alk1::URA3/alk1::HIS5*	His^+, Ade^+, Ura^+
DA123–1	DA23–8164	*his5, ade1, ura3, alk2-alk3::Δade1/alk2-alk3::Δade1 alk1::URA3/ALK1*	His^-, Ade^-, Ura^+
DA123–14	DA123–1	*his5, ade1, ura3, alk2-alk3::Δade1/alk2-alk3::Δade1 alk1::URA3/alk1::ADE1*	His^-, Ade^+, Ura^+
DA235–2	DA23–8164	*his5, ade1, ura3, alk2-alk3::Δade1/alk2-alk3::Δade1, alk5::URA3/ALK5*	His^+, Ade^-, Ura^+
DA235–24	DA235–2	*his5, ade1, ura3, alk2-alk3::Δade1/alk2-alk3::Δade1, alk5::URA3/alk5::ADE1*	His^-, Ade^+, Ura^+
DA235–243	DA235–24	*his5, ade1, ura3, alk2-alk3::Δade1/alk2-alk3::Δade1, alk5::ADE1/alk5::ADE1*	His^-, Ade^+, Ura^+
DA1235–1	DA236–284	*his5, ade1, ura3, alk2-alk3::Δade1/alk2-alk3::Δade1, alk5::ADE1/alk5::ADE1, alk1::URA3/ALK1*	His^+, Ade^+, Ura^+
DA1235–12	DA1236–1	*his5, ade1, ura3, alk2-alk3::Δade1/alk2-alk3::Δade1, alk5::ADE1/alk5::ADE1, alk1::URA3/alk1::HIS5*	His^+, Ade^+, Ura^+
DA1235–121	DA1235–12	*his5, ade1, ura3, alk2-alk3::Δade1/alk2-alk3::Δade1, alk5::ADE1/alk5::ADE1, alk1::HIS5/alk1::HIS5*	His^+, Ade^+, Ura^-

[a] Strains CHA1 (16), DA1–44 (17), and CHAU1 (18) were described previously.

(5FOA)[1] was added at a final concentration of 2 mg/ml. The *n*-alkane-assimilating property of the *C. maltosa* strains was tested both in the liquid and solid YNB media. The bacterial strain *Escherichia coli* MV1190 (Δ(*srl-recA*)306::Tn10(*tet*$_r$) Δ(*lac-pro*) *thi*$^-$, supE (*F'*, *proAB*, *lacI*$_q$, *lacZΔM15*, *traD36*)) was used for plasmid preparations and was grown in LB broth.

Plasmid Construction and Yeast Transformation for Gene Disruptions—A 1.8-kb *Hin*dIII-*Eco*T22I fragment of pCMU6 carrying the *C. maltosa URA3* gene (18) was subcloned into the *Hin*dIII-*Pst*I sites of pUC19 to construct pURAD. A 1.6-kb *Ssp*I-*Dra*III fragment of the *C. maltosa ADE1* gene (16) was cloned into the *Sal*I site of pUC119 through the *Sal*I linker to construct pUADE. A 6.7-kb *Sal*I-*Bam*HI fragment carrying entire *ALK2-B* and *ALK3-B* genes and their 5'- and 3'-noncoding regions (4) was subcloned into pUC19 to construct pUA23. For gene disruption of the *ALK2-ALK3* locus, a 1.6-kb *Eco*RV-*Bam*HI fragment of pURAD and a 1.5-kb *Dra*I-*Bam*HI fragment of pUADE replaced a 4.1-kb *Bgl*II-*Eco*RV fragment of pUA23 to construct pUD23U and pUD23A, respectively. The *alk2-alk3::URA3* and the *alk2-alk3::ADE1* cassettes for gene disruption were excised from pDA23U and pDA23A, respectively, with *Pst*I-*Bam*HI double digestions in both cases, and transformed into *C. maltosa*. The transformation of *C. maltosa* was performed by a modified lithium acetate method (14). For regeneration of the *ADE1* marker, pDA23Δade was constructed by ligation of a 1.3-kb *Pst*I-*Eco*RV fragment from pUD23A and a 1.3-kb *Eco*RV-*Bam*HI fragment from pUA23 into the *Pst*I-*Bam*HI sites of pUC19, and the *alk2-alk3::Δade1* cassette was generated by digestions with *Pst*I-*Bam*HI for the *C. maltosa* transformation. In these three cassettes, the complete *ALK2* coding region, NH_2-terminal one-third of the coding region of *ALK3*, and the *ALK2-ALK3* internal region (1.0 kb) were replaced.

A 2.1-kb *Hin*dIII-*Hin*dIII fragment of *ALK5-A* (6) was subcloned into the *Hin*dIII site of pUC19 to construct pUA5. A 0.6-kb *Bgl*II-*Eco*RV fragment of pUA5 was replaced with either the 1.6-kb *Eco*RV-*Bam*HI fragment of pURAD or the 1.5-kb *Dra*I-*Bam*HI fragment of pUADE to construct pUD5U and pUD5A, respectively. The *alk5::URA3* and the *alk5::ADE1* disruption cassettes were excised from pUD5U and pUD5A, respectively, with *Pst*I-*Sca*I digestions in each case. Both cassettes replaced the NH_2-terminal 40% of the coding region of *ALK5*.

A 2.8-kb *Eco*T22I-*Eco*T22I fragment of *ALK1-A* (3) was subcloned into the *Pst*I site of pUC119 to construct pUA1. A 0.5-kb *Eco*RV-*Eco*RV fragment corresponding to the central one-third of the *ALK1* coding region was replaced by a 1.8-kb *Hin*dIII-*Bam*HI fragment of pURAD containing *URA3* through blunt end ligation to construct pUD1U. The *alk1::URA3* disruption cassette was excised from pUD1U with *Sph*I-*Bam*HI digestions. The construction of the other plasmids for the *ALK1* disruption, pUD45A and pUD45H, was described previously (17).

Southern Blot Hybridization—Total DNA of *C. maltosa* was isolated from a culture of 10 ml of YNB-glucose medium as described (18). Southern blot analysis was performed with an ECL (enhanced chemiluminescence) gene detection system (Amersham) in accordance with the instruction of the supplier. For detection of the appropriate gene replacements of the *ALK2-ALK3* locus, total DNA from the transformants was digested with *Bam*HI and *Cla*I and probed with a 0.6-kb *Hin*dIII fragment of *ALK3-B* (see Fig. 2). For the *ALK5* and *ALK1* replacements, total DNA from the transformants was digested with *Hin*dIII and *Pst*I-*Bgl*II, respectively, and probed with a 2.1-kb *Hin*dIII fragment of *ALK5-A* and a 2.3-kb *Hin*dIII fragment of *ALK1-A*, respectively. The appropriate replacements were confirmed as follows. Both of the intact alleles of *ALK5* gave a 2.2-kb doublet band. The genomic blot of the first stop transformant gave a 3.3-kb band as predicted for the replacement with *URA3* in addition to the 2.2-kb band of the undisrupted allele. That of the second stop transformant gave new 1.0- and 2.0-kb bands as predicted for the replacement with *ADE1* in addition to the 3.3-kb band for the *URA3*-replacement, and the 2.2-kb band for the intact *ALK5* allele disappeared. In *ALK1* disruptions the genomic blots of the transformants for the replacements with *URA3*, *ADE1*, and *HIS5* gave 8.2-, 9.1-, and 9.8-kb bands, respectively. Disruption of both alleles of *ALK1* was confirmed by detection of each of the two respective bands for the replacements and by the disappearance of the 7.0-kb band for the intact allele of *ALK1*. A 0.9-kb *Sal*I fragment of pURAD (18), a 0.7-kb *Eco*RI-*Eco*RV fragment of *ADE1* (16), and a 2.3-kb *Eco*RI-*Hin*dIII fragment of *HIS5* (15) were also used as probes to confirm the replacements of *URA3*, *ADE1*, and *HIS5*, respectively.

P450 Induction Experiments—To determine the expression level of spectrally active P450 in the wild-type strain CHAU1 as well as in the disruptant strains DA235-24, DA123-14, and DA1235-12, respective cultures were first grown to a density of about 1×10^7 cells/ml in a yeast minimal medium containing 1.34% Yeast Nitrogen Base, 2% glucose and, as required, histidine (50 mg/liter), adenine (100 mg/liter), and uracil (40 mg/liter). Then cells were washed in the fresh medium without glucose and cultivated under the same conditions except for 1% dodecane as a sole carbon source. P450 contents were determined after an induction time of 4 h by means of CO difference spectra (10).

Plasmid Construction for Plasmid-based Complementation—A 1.8-kb *Pst*I-*Xho*I fragment of the TRA region, which contains an autonomously replicating sequence and a centromeric DNA of *C. maltosa* (19), was isolated from the vector pTRA11 (14) and cloned into the *Pst*I site of pUC18 through a *Pst*I linker. From the resulting plasmid a 1.8-kb *Pst*I fragment was inserted into the *Aat*II site of pUC19 through blunt end ligation. Then a 1.2-kb *Dra*I-*Xba*I fragment of *URA3* from pURAD was inserted into the *Xho*I site of the TRA region through an *Xho*I linker to construct pUTU1. A 2.8-kb *Bam*HI-*Sph*I fragment of *ALK1-A* isolated from pUA1 was inserted into the *Bam*HI-*Sph*I sites of pUTU1 to construct pUTU-ALK1. A 2.6-kb *Bgl*II fragment of *ALK2-A* and a 3.3-kb *Eco*T22I-*Eco*RV fragment of *ALK3-A* were inserted into the *Bam*HI site and the *Pst*I-*Sma*I sites of pUTU1 to construct pUTU-ALK2

[1] The abbreviations used are: 5FOA, 5-fluoro-orotic acid; kb, kilobase(s).

and pUTU-ALK3, respectively. A 3.5-kb *PstI-EcoRI* fragment of *ALK5-A* was inserted into the *PstI-SmaI* sites through blunt end ligation to construct pUTU-ALK5.

Exchanges of the promoter regions of *ALK1* and *ALK5* genes were done by polymerase chain reaction as follows. To obtain recombinant products having *ALK1* promoter and *ALK5* coding region, primers 5′-AACATCTGGTCATGATTGATGAAATACTTCCT-3′ and 5′-TCATCAATCATGACCAGATGTTATAAATG-3′ were used. For the product having *ALK5* promoter and *ALK1* coding region, primers 5′-TAAACTAAAATATGGCTATAGAACAAATTATTG-3′ and 5′-GTTCTATAGCCATATTTTTAGTTTAATCTTATTTAAA-3′, respectively, were used. Underlines correspond to the translational initiation codons. As flanking primers, M13 forward and reverse sequencing primers were used. Plasmids pUTU-ALK1 and pUTU-ALK5 were used as templates for the first polymerase chain reaction step to obtain the respective promoter and coding region. Then the second polymerase chain reaction step generated exchanged recombinant products. The products were cloned into the *KpnI-SphI* sites of pUTU1 to construct pUTU-A1/A5 (*ALK1* promoter and *ALK5* coding region) and pUTU-A5/A1 (*ALK5*-promoter and *ALK1*-coding region), respectively.

Heterologous Expression and Enzyme Assay—Construction of the four coexpression vectors used for the simultaneous production of the individual *C. maltosa* P450alk forms and NADPH-cytochrome P450 reductase was described previously (13). Heterologous expression in *S. cerevisiae* was done as described previously (11). The enzyme assay was carried out using the microsomal fraction using [1-^{14}C]dodecane (Sigma) and [1-^{14}C]hexadecane (Amersham) as substrates as described previously (13).

RESULTS

Disruption of Single Loci—We have shown that four out of the eight P450alk genes of *C. maltosa* are significantly induced by *n*-alkanes and may thus encode P450 enzymes directly involved in *n*-alkane assimilation in the previous study (6). These four genes have been designated *ALK1*, *ALK2*, *ALK3*, and *ALK5*. Each of them occurs in two allelic variants in agreement with the diploid nature of *C. maltosa* genome. Previous gene disruption experiments revealed that a *C. maltosa* strain defective in *ALK1* retained the ability to grow on long chain *n*-alkanes (17). Therefore, it has been the first objective of the present study to disrupt each of the other three *n*-alkane-inducible P450alk genes (for the experimental strategy applied, see Fig. 1) and to examine on this basis their contribution to the phenotype of *n*-alkane assimilation.

As a parental strain, *C. maltosa* CHAU1, in which three selectable auxotrophic markers (*ura3*, *ade1*, and *his5*) were available, was used (18). Taking advantage of the fact that *ALK2* and *ALK3* are clustered in about a 1.0-kb distance (4), these two genes could be disrupted simultaneously by a single gene replacement. Strain CHAU1 was first transformed with the *alk2-alk3::URA3* disruption cassette to uracil prototrophy. To disrupt the remaining intact allele of *ALK2-ALK3*, the resulting Ura$^+$ transformant was then transformed with the *alk2-alk3::ADE1* disruption cassette. The appropriate gene disruption was detected by Southern blot analysis of the genomic DNA of the first step Ura$^+$ transformant (strain DA23-8) and the second step Ura$^+$-Ade$^+$ transformant (strain DA23-81; see Fig. 2, *lanes 2* and *3*). The resulting strain DA23-81 (*his5*, *ade1*, *ura3*, *alk2-alk3::URA3/alk2-alk3::ADE1*) had disruption in both alleles of *ALK2-ALK3* locus. As in the case of the disruption of *ALK2-ALK3*, the disruption of *ALK5* was also performed using stepwise *URA3* and *ADE1* as selectable markers, thus generating the strain DA5-61 (*his5*, *ade1*, *ura3*, *alk5::URA3/alk5::ADE1*) from the parental strain CHAU1.

The growth phenotype of the strains DA23-81 and DA5-61 as well as the *ALK1*-disrupted strain DA1-44 (17) are shown in Table II. All of these strains were able to utilize at least medium and long chain *n*-alkanes (C14 and C16) as sole carbon sources for growth, indicating that none of the three loci (*ALK1*, *ALK2-ALK3*, and *ALK5*) was solely essential for *n*-alkane-assimilating ability of *C. maltosa*. However, it was obvious that

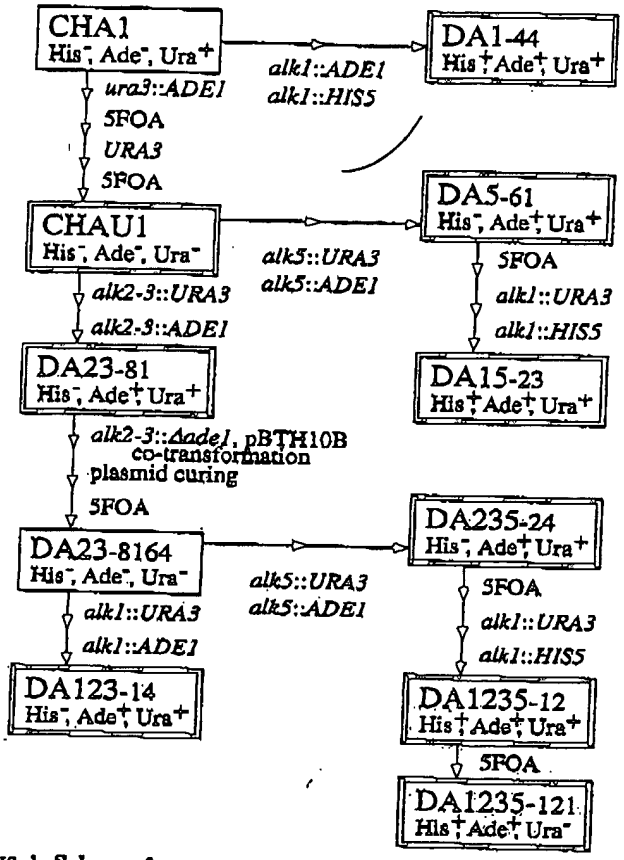

FIG. 1. Scheme for sequential gene disruption of the *C. maltosa* P450alk genes. The strains *boxed* in the *double line* were used to analyze their properties in the *n*-alkane assimilation. The appropriate genotypes were confirmed by Southern blot analysis of the isolated strains in every steps. The steps from strain CHA1 to CHAU1 (18) and from CHA1 to DA1-44 (17) were described previously. For details, see "Experimental Procedures."

the growth of the strain DA1-44 showed significant but weak growth on the *n*-alkane of short chain length (C12) compared with the parental and the other strains.

Regeneration of Selectable Markers—To investigate the cellular function of individual P450alk isoforms, the respective P450alk genes had to be disrupted simultaneously within a single strain. However, because the initial round of disruption resulted in a Ura$^+$ and Ade$^+$ phenotype and only the *HIS5* marker was available, methods to restore the selectable markers to the disrupted strains should have been developed to permit reutilization of the markers for further disruption of the remaining P450alk genes. In one method, the introduced selectable marker *ADE1* was substituted for a deletion derivative of it by means of cotransformation with a selectable plasmid to facilitate the selection of the replacement. The *ADE1*-deleting cassette *alk2-alk3::Δade1* was transformed into strain DA23-81 along with an *HIS5*-containing autonomously replicating plasmid, pBTH10B (15). Among the resulting His$^+$ transformants, some developed red colored colonies characteristic of the *ade1* mutant. They were tested for adenine auxotrophy, and the appropriate replacement with the *alk2-alk3::Δade1* cassette was confirmed by Southern blot analysis of the genomic DNA of an Ade$^-$ strain, DA23-816 (Fig. 2, *lane 4*). Then, the plasmid pBTH10B was cured from the strain DA23-816 by growth on nonselective medium.

The other methods used for the regeneration of a selectable marker in a single strain were based on the selectability of an

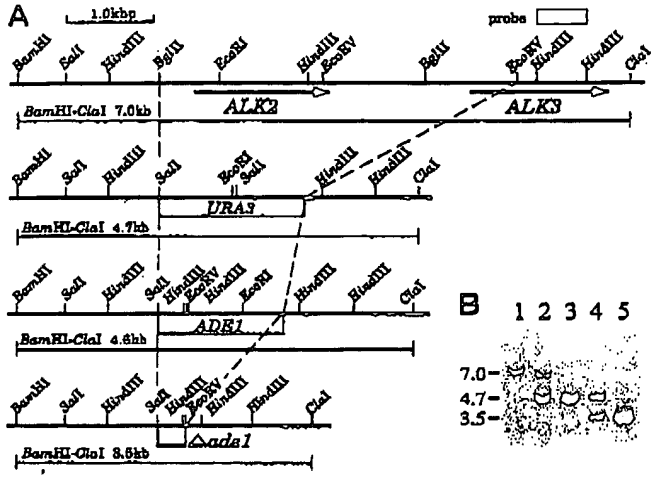

FIG. 2. **Gene disruption of *ALK2-ALK3* and regeneration of selectable markers.** *Panel A*, restriction maps are presumption from the gene disruption and regeneration experiments of the *ALK2-ALK3* region. The predicted lengths of the *Bam*HI-*Cla*I fragments are indicated. The *thick bar* indicates the probed region (the 0.6-kb *Hin*dIII fragment) used for Southern blot analysis. *Panel B*, the autogram shows Southern blot of genomic DNAs digested with *Bam*HI-*Cla*I. *Numbers* in the *left* of the autogram are the sizes of the fragments hybridized. *Lanes 1*, strain CHAU1; *lane 2*, DA23-8; *lane 3*, DA23-81; *lane 4*, DA23-816; *lane 5*, DA23-8164. The genomic blot of the strain CHAU1 gave only the 7.0-kb doublet band of both alleles of *ALK2-ALK3* (*lane 1*), whereas that of an Ura$^+$ transformant, strain DA23-8, gave a 4.7-kb band, as predicted for the replacement with the *alk2-alk3*::*URA3* in addition to the 7.0-kb band of undisrupted allele (*lane 2*). Hybridization of the second step Ade$^-$ transformant, strain DA23-81, revealed that the 7.0-kb band for the undisrupted allele of *ALK2-ALK3* had disappeared, and only the predicted bands for the replacement with the *alk2-alk3*::*URA3* and the *alk2-alk3*::*ADE1*, a doublet band of 4.7 and 4.6 kb, respectively, were detected (*lane 3*). The replacements with *URA3* in the 4.7-kb band (*lanes 2–4*) and with *ADE1* in the 4.6-kb band (*lane 3*) were confirmed by reprobing the same blot with *URA3* and *ADE1*, respectively (data not shown). The appropriate replacement of the *alk2-alk3*::*ADE1* allele with the deletion derivative of *ADE1* (Δ*ade1*) was confirmed in strain DA23-816 (*lane 4*). The predicted band for the replacement, 3.5 kb, was detected in addition to the 4.7-kb band for the *alk2-alk3*::*URA3* allele. When probed with *ADE1*, the 4.6-kb band of *alk2-alk3*::*ADE1* in the strain DA23-81 (*lane 3*) had disappeared in the strain DA23-816 (data not shown). Hybridization of a 5FOA-resistant and Ura$^-$ strain, DA23-8164, showed that the 4.7-kb band for the *alk2-alk3*::*URA3* allele had disappeared and that the 3.5-kb doublet band of the *alk2-alk3*::Δ*ade1* was detected. The 4.7-kb band for the *alk2-alk3*::*URA3* had also disappeared in strain DA23-8146 when probed with *URA3* (data not shown).

ura3 mutant by their resistance to 5FOA, a toxic analog of an intermediate in the uracil synthetic pathway. Application of the 5FOA selection of a *ura3* mutant in *C. maltosa* has already been shown in a previous report (18). Spontaneous 5FOA-resistant strains were selected from the strain DA23-816, and their uracil requirement was tested. Southern blot analysis of a Ura$^-$ strain, DA23-8164, indicated that this strain was homozygous for *alk2-alk3*::Δ*ade1* (Fig. 2, *lane 5*). As previously shown, a recombination event between both alleles occurred frequently during the selection of 5FOA resistance (18). The strain DA23-8146 (*his5*, *ade1*, *ura3*, *alk2-alk3*::Δ*ade1*/*alk2-alk3*::Δ*ade1*) was devoid of *ALK2-ALK3* in both alleles; nevertheless, three selectable markers could be utilized further in this strain.

Development of Multiple P450alk Gene Disruptions and Their Effects on n-Alkane Assimilation—Mutants containing multiple P450alk gene disruption were generated according to the scheme shown in Fig. 1. The complete set of disruption in all the three P450alk loci, *ALK1*, *ALK2-ALK3*, and *ALK5*, was obtained by sequential disruption. The complete genotypes of the strains generated are listed in Table I. In each step, the appropriate genotypes were confirmed by Southern blot analysis of the isolated strains (see "Experimental Procedures"), as shown in the case of the *ALK2-ALK3* disruption.

TABLE II
Growth properties of cytochrome P450alk gene disruptants on n-alkanes of different chain lengths

Strain	P450 form expressed	Carbon source		
		n-Dodecane	*n*-Tetradecane	*n*-Hexadecane
CHAU1	ALK1, ALK2, ALK3, ALK5	+	+	+
DA1–44	ALK2, ALK3, ALK5	+/−	+	+
DA23–81	ALK1, ALK5	+	+	+
DA5–61	ALK1, ALK2, ALK3	+	+	+
DA15–23	ALK2, ALK3	+/−	+	+
DA123–14	ALK5	−	+/−	+
DA235–24	ALK1	+	+	+
DA1235–12	None	−	−	−

The growth properties of P450alk gene disruptants on *n*-alkanes of different chain lengths as sole carbon sources were examined (see Table II). Strain DA1235-12, in which all four of the genes were disrupted, could not utilize *n*-alkanes of any chain lengths for growth, although this strain was able to grow on *n*-alcohols and fatty acids as sole carbon sources. The growth property of this strain indicated that the sequential gene disruption resulted in a complete functional block of the *n*-alkane assimilation at the first step.

Strains DA123-14, DA235-24, and DA15–23, in which two of the three loci were disrupted, showed good growth on C16, indicating that each of the products of the three loci was alone sufficient for the assimilation of long chain *n*-alkane. Interestingly, a specificity of the *n*-alkane-assimilating property was observed depending on the chain length of *n*-alkanes. The strain DA123-14 could not utilize C12 at all for growth, whereas the other strains grew significantly on this substrate, although some of them showed weak growth. And the strain DA123-14 alone showed weak growth on C14. This growth property of the strain DA123-14 indicated that the *ALK5* product that remained functional in this strain could function fully only on the long chain *n*-alkane. Strains devoid of *ALK1* showed merely poor growth on C12, whereas strains expressing *ALK1* showed good growth on C12. These results indicated that the *ALK1* product played an important role for assimilation of short chain *n*-alkanes.

To prove the expression of spectrally active P450 in the gene disruptant strains, P450s were induced by C12 as described under "Experimental Procedures." Whereas the wild-type strain CHAU1 expressed a significant amount of P450 (0.11 nmol/10^8 cells), no significant expression of P450 was observed in the P450 disruptant strain DA1235-12. Under the same conditions, the presence of P450 could be detected clearly in strain DA235-24 (0.09 nmol/10^8 cells) and DA123-14 (0.02 nmol/10^8 cells). Interestingly, in the strain DA235-24 in which only the *ALK1* product remained functional, almost 80% of the P450 amount of the wild-type strain could be found, indicating that this P450 form was most abundant P450 in *C. maltosa*. In contrast, the amount of P450 in the strain DA123-14 in which only *ALK5* remained functional was less than 20% of the wild-type strain.

Plasmid-based Complementation with Each P450alk Gene—Function of individual P450alk genes toward *n*-alkane assimilation was analyzed further by plasmid-based complementation. Each of four entire genes having 5′- and 3′-flanking regions was cloned into pUTU1, which carried both an autonomously replicating sequence and a centromeric DNA sequence of *C. maltosa* (19), transformed into the strain DA1235-121, which was devoid of all four of the genes and in which the

TABLE III
Plasmid-based complementation of the n-alkane assimilating properties of the strain DA1235-121

Plasmid	P450 form expressed	Carbon source		
		n-Dodecane	*n*-Tetradecane	*n*-Hexadecane
pUTU1	None	−	−	−
pUTU-ALK1	ALK1	+	+	+
pUTU-ALK2	ALK2	+/−	+	+
pUTU-ALK3	ALK3	+/−	+	+
pUTU-ALK5	ALK5	−	−	+
pUTU-A5/A1	ALK1	+	+	+
pUTU-A1/A5	ALK5	−	−	+

URA3-selectable marker could be utilized, and examined for *n*-alkane assimilating property. Table III summarizes the results of the complementation experiments. Complementation with *ALK1* resulted in the complete recovery of *n*-alkane assimilating property, to utilize *n*-alkanes of different chain length as sole carbon sources for growth. Although the disruption experiments could not distinguish between the function of the products of *ALK2* and *ALK3*, the results of the complementation indicated that both the *ALK2* and the *ALK3* products solely could function significantly in assimilation of *n*-alkanes of different chain length. However, both complemented strains grew only weakly on C12 as in the cases of the *ALK1*-disrupted strains. Unlike these three genes, *ALK5* was able to complement the assimilating property of *n*-alkanes with only C16 but not with C12 and C14.

To distinguish whether the functional specificity of the *ALK5* product depending on the chain length of *n*-alkanes was responsible for its enzymatic activity or for its expression level, two plasmids in which the promoter regions of *ALK1* and *ALK5* had been exchanged were examined for their ability to complement the assimilation phenotype of the strain DA1235-121 (see Table III). The strain carrying pUTU-A5/A1, which had the *ALK5* promoter region and the *ALK1* coding region, grew well on both the long and short chain *n*-alkanes. The strain carrying pUTU-1A/5A, which had the *ALK1* promoter region and the *ALK5* coding region, grew only on the long chain *n*-alkane, showing the same growth property of the strain carrying pUTU-ALK5A. The cellular P450 contents after induction with C12 were found to be nearly the same for both strains (0.03 and 0.02 nmol/10^8 cells, for strains carrying pUTU-A1/A5 and pUTU-A5/A1, respectively). These results indicated that although *ALK5* was induced by C12 to the significant amount in the cell, the presence of the *ALK5* protein itself did not contribute to the growth on C12, disclosing a restricted substrate specificity of the *ALK5* product toward C12.

Enzyme Activities of Individual P450alk Isoforms—Enzymatic activities of individual P450alk isoforms were characterized by means of turnover rates toward *n*-alkanes. Because expression levels of P450 in the complemented strains were limited, heterologous overexpressions of individual P450alk forms in another yeast, *S. cerevisiae*, in which the related activity is absent, were applied. A highly active P450 monooxygenase reconstitution system consisting of P450alk and NADPH-cytochrome P450 reductase from *C. maltosa* has been established already *in vivo* in *S. cerevisiae* (11, 13). Microsomal fractions were prepared from the respective strains overexpressing P450alk and then assayed for P450alk-encoded hydroxylase activities toward C12 and C16 (see Table IV). The *ALK1* product displayed the strongest hydroxylase activity, suggesting that it was the most important P450alk form for the primary hydroxylation of *n*-alkanes. Products of *ALK2* and *ALK3* showed *n*-alkane hydroxylation activities of approximately half of the *ALK1* product, respectively. No preference of the activity depending on *n*-alkane chain lengths was observed

TABLE IV
Activity of C. maltosa cytochrome P450alk isoforms toward n-dodecane and n-hexadecane

Heterologous overexpression of individual P450alk forms in *S. cerevisiae* yielded 60–115 (pmol/10^8 cells) P450. Microsomal fractions containing 160–380 (nmol/mg of protein) P450 and 2.5–8.2 (nmol/mg of protein/min) cytochrome *c* reductase activity were used for measuring *n*-alkane hydroxylation activity. Values given in parentheses indicate numbers of the experiments done. The hydroxylation data determined here confirmed the turnover rates of the *C. maltosa* cytochromes P450 known so far (13).

P450 form	Activity		Ratio (C_{12}/C_{16})
	n-Dodecane (C_{12})	*n*-Hexadecane (C_{16})	
	nmol product/nmol P450/min		
Alk1	44.0 ± 3.2 (3)	48.0 ± 3.0 (4)	0.92
Alk2	25.5 ± 1.5 (3)	22.0 ± 1.9 (4)	1.16
Alk3	26.0 ± 3.3 (3)	23.2 ± 2.7 (5)	1.12
Alk5	1.8 ± 0.5 (3)	11.6 ± 3.3 (6)	0.16

for these three P450alk forms. The *ALK5* product showed relatively weak but significant activity toward C16 (approximately one-forth of the *ALK1* product), whereas it showed very little activity toward C12. The substrate specificity of the *ALK5* product toward the long chain *n*-alkanes was congruent with the results of the sequential gene disruption.

DISCUSSION

To our knowledge, this is the first report about gene disruption of multiple forms of cytochromes P450 and about its direct effect on phenotype. The sequential gene disruption of the P450alk genes resulted in a *C. maltosa* strain (DA1235-12) that could not utilize *n*-alkanes as sole carbon sources for growth because of the lack of the functional P450alk. Because only the P450alk genes were manipulated in the genome, this result provides direct evidence that P450alk participates in the *n*-alkane assimilation pathway. In the proposed *n*-alkane assimilation pathway, *n*-alkane is first hydroxylated at the terminal position to produce *n*-alcohol and then oxidized successively to fatty acids. The growth property of the disrupted strain that could not assimilate *n*-alkanes but could grow on *n*-alcohols and fatty acids as sole carbon sources clearly correlated with the *in vitro* *n*-alkane-hydroxylating activity of the P450alk enzymes shown here and in previous studies (5, 9, 12, 13), confirming that P450alk catalyzes the first step of *n*-alkane assimilation. This growth property also indicates that none of the four isoforms analyzed is essential in the further downstream steps of the assimilation pathway, although considerable fatty acid ω-hydroxylation activity has been shown for some of them (5, 11–13).

The overlapping function of the four P450alk forms toward hydroxylation of at least long chain *n*-alkanes is supported by the results that disruption of all four of the genes was necessary to generate the *n*-alkane-nonassimilating strain and that each of the four genes could complement the assimilating ability of the disrupted strain. Furthermore, their *n*-alkane hydroxylation activities were confirmed directly by an *in vitro* assay using a heterologous overexpression system.

The *ALK1* product is the most important P450alk isoform because when it was present in the cells, the growth was significant on *n*-alkanes of any chain length. Its functional importance was supported by the strongest *in vitro* enzyme activity among the P450alks examined as well as by the considerable amount in the cells as shown in the strain DA235-24 in which only the *ALK1* product was the functional P450alk. The absence of the functional *ALK1* product in the series of disruptants resulted in weak growth on the short chain *n*-alkanes, indicating that the *ALK1* product is not essential but necessary for full activity for the assimilation of short chain

n-alkanes. Because the previous analysis of the gene disruption of *ALK1* had been done using only *n*-hexadecane (17), we could not find this importance. The *ALK2* and the *ALK3* products can also hydroxylate *n*-alkanes of any chain length, although their function on short chain *n*-alkanes is restricted. However, the *ALK5* product demonstrated a narrow spectrum toward long chain *n*-alkanes. The *in vivo* complementation experiment using the promoter-exchanged constructs and the direct *in vitro* enzyme assay (see Tables III and IV) showed that the functional specificity was affiliated not to the expression level of the enzyme but to the enzyme activity itself.

Previous analysis on the induction of individual P450alk genes (6) revealed that *ALK1* is induced strongly by short chain *n*-alkanes but relatively weakly by long chain *n*-alkanes. This result is congruent with the functional importance of the *ALK1* product on short chain *n*-alkanes. However, *ALK5* is induced by both long and short chain *n*-alkanes, whereas its product shows the substrate preference toward long chain *n*-alkane. Furthermore, although the four P450alk isoforms are not essential for the following steps of *n*-alkane assimilation pathway, three genes, *ALK1*, *ALK2*, and *ALK5*, are induced weakly but reproducibly by *n*-alcohols and fatty acids. The regulation of the expression of individual P450alk forms is not simply correlated with their functions.

The P450alk gene family in *C. maltosa* consists of at least eight members (6). The four isoforms characterized here are significantly induced when the carbon sources are *n*-alkanes, whereas the other four genes (*ALK4*, *ALK6*, *ALK7*, and *ALK8*) are induced only at low levels by *n*-alkanes as well as *n*-alcohols and fatty acids. The growth property of the *n*-alkane-nonassimilating strain indicates that these undisrupted four P450alk genes cannot function fully on the assimilation. However, further analysis such as *in vitro* assay using heterologous overexpression is necessary to determine whether they have enzymatic activity toward *n*-alkanes.

Methods to restore the auxotrophic requirements to cells previously transformed to respective prototrophy with the selectable marker genes have been developed here in *C. maltosa* to allow sequential gene disruption within a single strain. According to the strategy used previously (17), only both alleles of a single gene could be disrupted in a stepwise manner using two selectable markers. The cotransformation with a selectable autonomously replicating plasmid facilitated the selection of the regeneration of the *ade1* auxotrophy. Moreover, the strain regenerating the *ade1* marker could be distinguished easily because of red color development of the colony. Although the condition for the cotransformation was not optimized, a frequency of one *ade1* strain among approximately 300 His^+ transformants was obtained. Such a cotransformation strategy has also been applied in *C. albicans* where the gene replacement was selected by colony hybridization (20).

The 5FOA selection method also allowed us to regenerate the *ura3* marker by means of the positive selection of a *ura3* mutant. The strain having heterozygous disrupted alleles, one replaced with *URA3* and the other by another marker or its deletion derivative, was rendered homozygous through mitotic recombination. Such a recombination was observed frequently in our previous study, in which *ura3* mutants homozygous for disruption were obtained from the strain heterozygous for disruption by selecting the 5FOA resistance (18). Almost all *ura3* mutants isolated by the 5FOA selection in this and in the previous studies were homozygous for disruption with a few exceptions, in which a local mutation in the *URA3*-carrying allele occurred. Such a strategy for the disruption of both alleles of a single gene has been applied previously to *C. albicans*, where UV irradiation has been used to facilitate recombination (21). The repeated use of *URA3* for sequential disruptions has also been reported in *C. tropicalis* (22). In the case of *C. tropicalis*, since only *URA3* has been available as a selectable marker in the host strain, the regeneration required the nystatin enrichment of *ura3* strains. In principle, the combination of the two methods for regenerating the auxotrophic markers would allow us disruption of a nonlimiting number of genes in *C. maltosa*.

The disrupted strain unable to assimilate *n*-alkanes may provide a useful host for further investigation of structure-function relationships of P450alk because mutant-type P450alks, the wild-type of which is nonfunctional on a specific substrate, can be positively selected by its *n*-alkane assimilating phenotype if they are functional. Because the structures of the four isozymes are significantly similar (56–67% amino acid identities) to each other (4, 6), P450alk is a suitable model for such a study. Industrially, *C. maltosa* may also be useful for the production of some hydrophobic metabolic intermediates produced by a selected P450alk form such as dicarboxylic acids. Some P450alk isoforms should play important roles for the production because considerable fatty acid ω-hydroxylation activity has been reported for them (5, 11–13). In combination with the functional block of the β-oxidation system by gene disruption as described in *C. tropicalis* (22, 23) and *C. maltosa* (24) and gene disruption of the appropriate P450alk forms as described here, it may be possible to construct an efficient strain for production.

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